# THE ANTIMITOTIC AND CARCINOSTATIC ACTION OF OVARIAN EXTRACTS [1]

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The search for chemical substances which might have a retarding effect on the growth of tumors has led along many paths. All sorts of substances have been tried. Those investigators who have theorized at all have for the most part thought in terms of some block of metabolic activity. Our own program has sought to discover relatively non-toxic antimitotic substances of natural origin, and in the search for such substances we have based our attack on what we believe to be a proper theory for the initiation and suppression of mitosis. Such a theory is discussed in some detail in a recent book (Heilbrunn, 1956). It holds that the mitotic spindle results from a gelation of the protoplasm, the mitotic gelation. Various substances can prevent this gelation by keeping protoplasm fluid. And because the most usual type of protoplasmic gelation and the type involved in the mitotic gelation is a clotting similar to the clotting of blood, it is our belief that anticlotting agents such as heparin or similar substances can prevent cell division. This indeed they do. The protoplasmic colloid contains substances which favor clotting and those which tend to prevent it. We have made extracts from various tissues and have found that ovaries are especially rich in anticlotting agents. These appear to resemble heparin and to be mucopolysaccharides. This work (Heilbrunn, Wilson and Harding, 1951; Heilbrunn, Chaet, Dunn and Wilson, 1954; Heilbrunn and Wilson, 1956) showed that the ovaries of various invertebrates and fishes do actually contain antimitotic substances which prevent the mitotic gelation and suppress cell division. In the search for some substance or substances which might eventually prove to have clinical value, we have recently investigated the ovaries of mammals and especially large mammals. In what follows, we will attempt to show first that extracts of mammalian ovaries do have antimitotic action, an action which is associated with a liquefying or anticlotting effect on the protoplasm; and second that such extracts may possess carcinostatic activity.

## Materials and Methods

In studying antimitotic activity, the most favorable test objects and the easiest to work with are the eggs of various marine invertebrates. Eggs such as sea urchin eggs or those of the marine worm Chaetopterus divide synchronously following fertilization. These eggs can be obtained in large quantity and they represent a surprisingly constant material. In previous studies on Chaetopterus eggs, we have described the simple techniques required (Heilbrunn and Wilson, 1948). Most of

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our work was done on Chaetopterus eggs. One experiment was done on eggs of the clam Spisula. The technique required for the use of this egg has been described by Allen (1953). All of our experiments were done at a controlled temperature of 21° C. Two minutes after insemination the eggs were placed in the solutions to be tested.

Crude extracts were prepared by cutting the ovaries into thin slices and immersing these slices in solutions of acidified sea water at a pH of approximately 4.8. For each gram of tissue, one ml. of sea water was used. After extraction the extract was brought to the pH of sea water by the addition of NaOH. In a few cases the ovaries were homogenized before extraction, but this seemed to produce less favorable results in our antimitotic studies. The acidification of the sea water used in extraction was apparently not necessary and potent antimitotic extracts could be obtained over a wide range of pH values.

The Chaetopterus egg has a very fluid protoplasm which stiffens markedly before the formation of the mitotic spindle. There is indeed a two-fold increase in the viscosity of the protoplasm and this can readily be followed with the centrifuge method described in earlier studies. With a simple hand centrifuge, turned so as to give a force approximately 2250 times gravity, for some time after fertilization it requires only 7 seconds to move granules through the protoplasm of the Chaetopterus egg to such an extent as to give the appearance of zones. The number 7 is taken as a relative value for the viscosity of the protoplasm; actually it probably represents about twice the value of the viscosity in centipoises. At approximately 30 minutes after fertilization, 14 seconds of exposure to a force 2250 times gravity is necessary to produce the appearance of zones in the protoplasm. In studying the antimitotic and the anticlotting action of our extracts we determine the percentages of cleavage following exposure to various dilutions, and we also determined the effect of the extracts on the viscosity of the protoplasm at the time when the normal control eggs showed increased viscosity. These tests had to be made rapidly, for the duration of the mitotic gelation is short.

Crude extracts prepared in the way we have indicated are very potent anticlotting and antimitotic agents, as our results will show, but generally speaking they are not very effective as carcinostatic agents. Indeed these extracts contain not only substances which tend to prevent protoplasmic clotting, they also contain substances which have exactly the opposite effect. Thus, although in one case we did obtain a definite carcinostatic effect with such crude extracts, for the most part we were not successful, and indeed in some instances the survival time of cancerous mice treated with crude extracts was decreased rather than increased. Hence we were led to try and find methods of extraction which would give us preparations of as high a carcinostatic action as possible with a minimum of toxicity or adverse action.

For many months, we had little or no success. Then we hit on a method of fractional alcoholic precipitation, and this has now provided us with extracts which possess a definite carcinostatic action. This was perhaps to be expected, for in studying the antimitotic action of extracts of starfish ovaries, we found that the potent substance could be precipitated by alcohol (Heilbrunn, Wilson and Harding, 1951).

The procedure we finally adopted was the following: Cow ovaries, fresh from the slaughter-house, were ground up in a meat grinder and were then extracted

in a solution containing 0.9% sodium chloride and 0.125% sodium bicarbonate. For each gram of ovarian material, 2 ml. of solution were used. The extraction was carried out in a cold room at 5° C. with constant stirring, and was continued for 16–18 hours. After the extract was strained through cheesecloth, it was centrifuged at 2000 rpm in a refrigerated centrifuge. The supernatant was then centrifuged in a Spinco centrifuge at 16,000 rpm (20,000 g) for an hour. The resultant supernatant was then precipitated by various concentrations of alcohol at approximately 0° C. Following each precipitation by a given percentage of alcohol, the supernatant was decanted. The precipitates were then lyophyllized. Our final product represented only a small fraction of the original ovarian material. Thus typically by precipitation with 45–60% ethanol we obtained about 10 mg. from a kilogram of ovaries.

In studying carcinostatic action, we used Swiss white mice. These were inoculated routinely with 1,500,000 Ehrlich ascites tumor cells in a volume of one ml. of ascitic fluid. Then 24 hours after inoculation, treatment with the extracts was begun. Each day for five days, each one of the mice to be treated was injected with a solution containing 15 mg. of the material. In general the 15 mg. were dissolved in 0.5 ml. of saline solution. Details of our technique and growth studies of our particular tumor will be published in a paper to be written by two of us (Tosteson and Davidson).

## Results

In the summer of 1955, we tested the effect of crude extracts of mammalian ovaries on cell division in the Chaetopterus egg. These extracts in every case stopped cell division. They also prevented the mitotic gelation. Our results are shown in Table I. In most cases the viscosity of the protoplasm at 30–40 minutes after fertilization is given as "less than 8." It may have been decidedly less than 8, but in the short space of time available for these measurements it was not possible to make enough tests to be sure of an exact value. However, the fact that the viscosity was less than 8 is a sure indication that the mitotic gelation has been suppressed, for during this gelation, the viscosity rises to a value of 14.

In addition to the data presented in the table, we have results from a few additional experiments; these results are entirely consistent with those shown in the table. In most of these other experiments, we attempted to improve the potency of the extracts by purifying them, but in every case the crude extracts prepared as described in the previous section were superior to the "purified" product. We also tried modifying our extraction procedures. In the results reported in the table, the extractions were made at a pH of 4.8, but we also tried extracting the ovarian material at pH's of 2.25, 3.90, 6.15, 7.1, and 10.0. All of the extracts prepared at these different pH's were likewise effective. Heating the extracts did not seem to have a very harmful effect, although our results were somewhat variable and minor differences in procedure seemed to be important. In one experiment an extract kept at 100° C. for three hours was still highly potent. In other experiments, exposure to 100° C. for shorter periods caused some loss in potency. Extracts made in distilled water and then lyophyllized were not effective. When the ovaries were homogenized before being extracted, the results were less favorable. Homogenization seemed to favor the release of thromboplastic substances into the extraction medium.

Although during the summer of 1955, we were never able to obtain any degree of successful purification of our extracts, in the following winter one of us (R. J. Rutman) hit upon the plan of precipitation with various concentrations of alcohol. Some of the precipitates obtained in this way when dissolved in saline solution had a very definite carcinostatic action, and we determined therefore to test the anti-mitotic action of these precipitates. Chaetopterus eggs (at Woods Hole) are only available during the summer months, so that we had to wait until the summer of 1956 before making our tests.

In making these tests, we had some difficulty, for the precipitates dissolved scarcely at all in the sea water in which we had to use them. However what tests we were able to make with the limited amount of material we had at our disposal indicated that only those precipitates obtained with intermediate concentrations of alcohol were effective. We had three fractions, of which A represented the precipitate obtained from cow ovary extracts with 0–45% alcohol, B the precipitate obtained with 45–60% alcohol, and C the precipitate with 60–80% alcohol. Of these three fractions, neither A nor C was very soluble. Fraction B seemed to go into solution, but when the solution was looked at under the microscope it was seen to have a large number of small solid particles suspended in it. We did several experiments with this fraction B. In one of these experiments, 5 mg. were dissolved in one ml. of sea water and then this solution was diluted so that the resultant dilutions contained 2.5 mg. per ml. of sea water and 1.25 mg. per ml. of sea water, respectively. All three of these solutions prevented the mitotic gelation and in all of them the fluidity of the protoplasm of the Chaetopterus eggs was maintained. In the most concentrated solution only 29% of the eggs cleaved, in the middle concentration 19%, and in the most dilute of the three solutions, 26% of the eggs cleaved. In the control 90% of the eggs cleaved. These counts were made 30 minutes or more after 50% of the control eggs had cleaved. The fact that the

TABLE I

*Effect of ovarian extracts on the mitotic gelation and on cleavage of the eggs of Chaetopterus*

| Source of material | Duration of extraction in minutes | Dilution | Viscosity at 30–40 min. | % cleavage | % cleavage in control |
|---|---|---|---|---|---|
| Cow | 30 | — | 4 | 1 | 98 |
| Cow | 60 | — | 4 | 0 | 98 |
| Cow | 120 | — | less than 8 | 0 | 100 |
| Cow | 120 | $\frac{1}{2}$ | less than 8 | 0 | 100 |
| Cow | 120 | $\frac{1}{4}$ | about 8 | 0 | 100 |
| Cow | 120 | $\frac{1}{8}$ | about 8 | 5 | 100 |
| Cow | 60 | — | less than 8 | 0 | 99 |
| Cow* | 60 | — | 5 | 0 | 99 |
| Cow** | 60 | — | 5 | 0 | 99 |
| Cow | 120 | — | less than 8 | 0 | 99 |
| Cow | 120 | — | less than 8 | 0 | 100 |
| Pig | 155 | — | less than 8 | 0 | 99 |
| Lamb | 105 | — | less than 8 | 0 | 99 |

* Ovarian capsules.
** Corpora lutea.

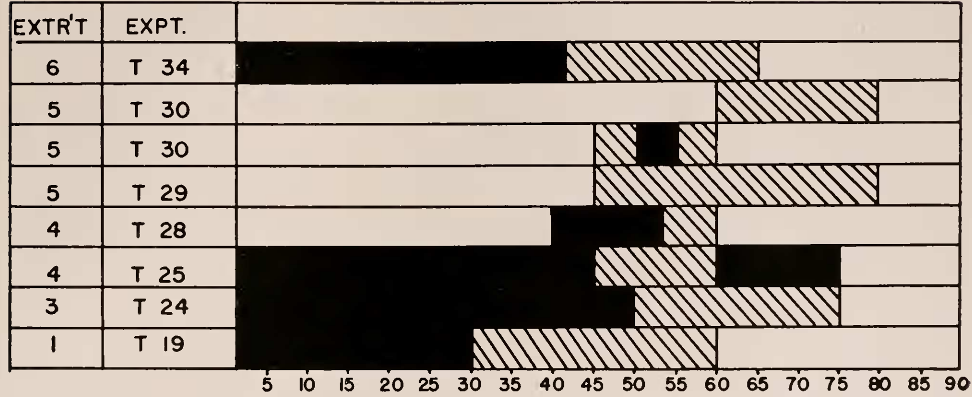


FIGURE 1. Activity of cold alcohol precipitates of aqueous extracts of ovarian tissue (cow).

various concentrations of material all acted in essentially the same way indicates that the material was only slightly soluble so that the actual concentration in true solution was the same in all three cases. In the very few experiments that we did with fractions A and C, these were without effect, perhaps because their solubility was extremely low.

In addition, the material of fraction B was tried on the eggs of the clam Spisula, and in this case also the material exerted an antimitotic effect. Concentrations of 2.5 mg. per ml., 1.25 mg. per ml. and 0.625 mg. per ml. showed strong antimitotic action, the cleavage being reduced from a control value of 97% to values of 31% for what presumably was the more concentrated solution, 39% for the intermediate concentration, and 40% for the weakest concentration.

Thus in spite of the fact that the material was soluble only slightly, the fraction obtained by precipitation with 45–60% alcohol did actually exert an antimitotic action, and in the case of Chaetopterus eggs this action was associated with a prevention of the mitotic gelation. No attempt was made to study the effect of fraction B on the mitotic gelation in the Spisula egg, for as yet the cycle of viscosity changes in the Spisula egg has not yet been worked out with sufficient thoroughness.

Clearly, our results with marine eggs indicate that fractionation of the crude cow ovary extracts by alcoholic precipitation can preserve the antimitotic and antigelating action.

Let us consider now the carcinostatic action of these alcoholic precipitates obtained from extracts of cow ovaries. Up until the present, we have accumulated a

large body of data. Indeed we have experimented with well over 4,000 mice. Our results show that the precipitates obtained by treating cow ovary extracts with intermediate concentrations of alcohol can cause survival of some 15–25% of mice previously inoculated with a lethal tumor, a tumor which regularly kills 100% of all mice properly inoculated with it.

Figure 1 illustrates the fact that treatment with intermediate concentrations of alcohol in the cold can produce precipitates which have a carcinostatic action. This figure gives the results obtained with five different extracts and is based on experiments with about 2,000 mice. Further details of these experiments will be presented in another paper soon to be prepared by several members of our group (Tosteson, Davidson and Rutman). Since the data for the table were collected we have obtained additional confirmatory data. Also we have been experimenting with various other types of extraction media and with other types of fractionation. Preliminary results lead us to the hope that extracts and fractions can be obtained which will cause a higher percentage of survival than we have been able to obtain with the extracts described in this paper.

## Discussion

Our work has been based on the idea that the protoplasm of all cells is much alike both chemically and physically, that inasmuch as the process of mitosis is much the same throughout the animal kingdom, the forces and agents which initiate cell division and those which suppress cell division are also much the same. On the basis of this pattern of thought, and on the basis also of many experiments on the initiation and suppression of mitosis (see Heilbrunn, 1956), we have been able to develop a new type of carcinostatic agent. At present this agent is at least as potent for our Ehrlich ascites tumors as other long-studied agents. It is our hope that the type of agent we are using can be perfected to give even better results. This will require much additional work.

## Summary

Extracts of the ovaries of cows, pigs and sheep can suppress mitosis in eggs of the worm Chaetopterus. This they do by keeping the protoplasm fluid and inhibiting the mitotic gelation which is a necessary precursor of the mitotic spindle. The potent substance or substances in extracts of cow ovaries can be precipitated by treating the extracts in the cold with intermediate concentrations of alcohol. Such purified preparations have a definite antimitotic effect and they also have a very definite carcinostatic action.

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